

*Meeting Summary*

**National Institutes of Health Workshop:  
The Promise of Induced Pluripotent Stem Cells (iPSCs)**

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**Welcome and Opening Remarks**

*Story C. Landis, Ph.D.; National Institute of Neurological Disorders and Stroke, National Institutes of Health (NIH)*

Dr. Landis began by thanking attendees for their willingness to discuss this rapidly-evolving field of research. She noted that the literature on induced pluripotent stem cells (iPSCs) has suggested potential for these cells in numerous applications of basic research, disease modeling, and future therapies. However, she also observed that the field is emerging, and this workshop is designed to provide a forum to discuss cutting-edge iPSC research and to illuminate the central issues that currently challenge the field. She noted that the recent Federal stimulus package will support many grants to study these cells, and the NIH seeks a clear understanding of the promise of these cells as well as the barriers that iPSC researchers currently face. As such, the workshop spans a range of topics that will highlight current research findings and discuss issues of concern to the field.

Dr. Landis also noted that a draft version of the proposed NIH guidelines for Federal support of human embryonic stem cell (hESC) research is available for public comment until May 27. The NIH is currently analyzing and categorizing comments on these guidelines as they are received. She noted that the Institutes seek thoughtful comments on how the proposed guidelines will meet the needs of the research community to support responsible, useful research.

**Session 1: Comparison of hESCs and iPSCs**

**Moderator:** *Story C. Landis, Ph.D.; National Institute of Neurological Disorders and Stroke, National Institutes of Health (NIH)*

**Controlling Stem Cells**

*Ronald McKay, Ph.D.; National Institute of Neurological Disorders and Stroke, NIH*

Dr. McKay began by observing that the research community is interested in the potential of using iPSCs as a source of functional somatic cells. Currently, the evidence indicates that functioning somatic cells, including mouse dopamine neurons (Lee SH, et.al. *Nat Biotechnol* 2000;18:675-679; Kim DS, et.al. *J Neurosci* 2002;22:9841-9849), mouse islets (Lumelsky N, et.al. *Science* 2001;292:1389-1394) and functional human islets (Shim JH, et.al. *Diabetologia* 2007;50:1228-1238), can be generated. For this type of technology to be effective, however, it is critical to understand how iPSCs behave. Cells from the mouse are similar to human cells and provide a benchmark for understanding. Recently, cell lines from the mouse epiblast have been used to generate stem cells, called post-implantation epiblast-derived stem cells (EpiSCs), that are distinct from mouse ES cells in their epigenetic state and the signals that control their differentiation (Tesar PJ, et.al. *Nature* 2007;448:196-199). Although the two types of mouse stem cell lines are similar in many ways, they use different transcriptional regulatory networks. For example, while the mouse ES cell requires the Jak/STAT system to differentiate, inhibition of this system facilitates differentiation of both the mouse epiblast stem cells and of human ESCs (hESCs). Mouse ES cells can be directed through the EpiSC stage to create differentiated somatic cells. ES cells express many genes seen in the germ line and in gametes that are shut down in the EpiSC state, although these may be reactivated using appropriate signals.

To unlock the potential of reprogrammed cells, it is critical to understand the principal cell types of the early embryo. The efforts of many researchers have laid the foundations for this understanding, using a mouse model. For example, work from Janet Rossant's laboratory has shown that extra-embryonic cell types can be isolated as stable cell lines, and Niels Geijsen and colleagues have demonstrated that stable cells are distinct from inner cell mass ES cells and epiblast cells. Austin Smith and coworkers have shown that a single gene controls movement of from the epiblast cell to the ES cell, and Azim Surani and colleagues have demonstrated that epiblast cells can also be differentiated into germ cells *in vitro* and *in vivo*.

Human ES cells grow as colonies, the formation of which depends on the density of the fibroblast feeder layer. Cluster analysis of the genomes of ES and iPS cells indicates similarities, although the cell types are not identical. However, at present, the differences have not been characterized fully. Similar gene expression patterns are seen in the epiblast-derived cells created by Tesar and colleagues (*Nature* 2007;448:196-199) and the iPSCs created by Yamanaka and coworkers (Takahashi K, et.al. *Cell* 2007;131:861-872). The similarity in the overall gene expression patterns is encouraging, although additional basic research is needed to understand how these cells differ. Several genes are of particular interest to these studies. For example, it has been shown that a single nucleotide polymorphism in the O(6)-methylguanine-methyltransferase (MGMT) promoter, *MDM2*, attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans (Bond GL, et.al. *Cell* 2004;119:591-602). Methylation status of the MGMT promoter predicts the response of malignant gliomas to alkylating agents, and homogenous MGMT immunoreactivity correlates with unmethylated MGMT promoter status in brain metastases of various solid tumors (Ingold B, et.al. *PLoS ONE* 2009;4:e4775). The epigenetic regulation of X-inactivation must also be explored more fully, as there is extensive variability in the X-inactivation profile in females (Carrel L, Williard HF. *Nature* 2005;434:400-401). Also, polymorphisms in genes such as glutathione S-transferase theta-1 cause variability (some ES cells delete the gene) that must be investigated further.

Dr. McKay concluded with a suggested mission for the upcoming two years, which entails analyzing more than 100 pluripotent cell lines and more than 50 derivative human cell lines at high resolution (e.g., DNA sequencing, RNA, proteomic analyses). He noted that while iPSCs hold great promise, much work is needed to characterize cell lines and to understand the mechanisms by which these cells operate.

#### Discussion:

Attendees discussed potential areas of focus for the NIH to consider in the upcoming few years. Participants debated whether detailed analysis of the iPS cell lines was necessary. It was noted that precise details of the individual lines would be needed in the event that a cell line was tested in a clinical trial. The number of iPS cell lines that could be characterized thoroughly will depend on the logistics of growing the cells and the knowledge of the human genome. Another attendee mentioned the need to understand the changes imposed on the cells by physiologic stresses, such as viruses. Cells selected and cultured in an unstressed environment may perform differently when inserted into a stress-laden environment. It was also observed that a rigorous analysis is needed on all iPSC-derived cells.

***Understanding Nuclear Reprogramming into iPS Cells****Konrad Hochedlinger, Ph.D.; Massachusetts General Hospital*

Dr. Hochedlinger began by defining nuclear reprogramming as an experimental way to induce mitotically stable changes in a cell's identity. These changes are usually associated with the reacquisition of a pluripotent state, thereby endowing the cell with developmental potential. Methods to achieve reprogramming include somatic cell nuclear transfer (SCNT), cell fusion, and *in vitro* reprogramming. Data in mice suggest that SCNT techniques give rise to reprogrammed cells that are essentially the same as ES cells. However, cell fusion, which fuses somatic cells with ES cells, creates pluripotent hybrid cells that are ultimately not viable for treating disease. *In vitro* reprogramming, which involves adding a series of transcription factors to somatic cells, creates iPSCs. It has been shown that manipulation of transcription factors in different contexts can change one cell type to another. For example, manipulation of transcription factors can enable iPSCs to become trophectodermal or endodermal cells. Similarly, over-expressing transcription factors in adult cells can enable transdifferentiation, or the reprogramming of mature cells into different types of mature cells.

The introduction of a few transcription factors (usually three or four) into a somatic cell can reprogram the cell into an iPSC after several weeks in culture (Takahashi K, Yamanaka S. *Cell* 2006;126:663-676; Maherali N, et.al. *Cell Stem Cell* 2007;1:367-368; Wernig M, et.al. *Cell Stem Cell* 2007;2:10-12; Okita K, et.al. *Nature* 2007;448:313-317). These cells are highly similar to ES cells. However, the process is slow and inefficient; only 0.01% - 1% of fibroblasts reprogrammed using *Oct4*, *Sox2*, *Klf4*, and *c-Myc* convert to iPSCs (Stadtfield M, et.al. *Cell Stem Cell* 2008;2:230-240; Brambrink T, et.al. *Cell Stem Cell* 2008;2:151-159). Successful reprogramming depends on the cellular context. For example, fibroblasts require more time for factor expression and have lower efficiency of reprogramming than do human keratinocytes or mouse neural stem cells (Stadtfield M, et.al. *Cell Stem Cell* 2008;2:230-240; Brambrink T, et.al. *Cell Stem Cell* 2008;2:151-159; Maherali N, et.al. *Cell Stem Cell* 2008;3:340-345; Aasen T, et.al. *Nat Biotechnol* 2008;26:1276-1284; Scholer HR. *Biol Chem* 2008;389:789). Recent work has suggested that reprogramming of stem or adult progenitor cells may result in approximately a 200-fold increase in efficiency compared to mature cells.

Reprogramming technology has the potential to treat many diseases, including Alzheimer's disease, Parkinson's disease, diabetes, and amyotrophic lateral sclerosis. In theory, easily-accessible cell types (such as skin fibroblasts) could be biopsied from a patient and reprogrammed, effectively recapitulating the patient's disease in a culture dish. Proof-of-principle experiments have shown that patient-specific iPSCs can be derived. (Dimos JT, et.al. *Science* 2008;321:1218-1221).

While ES cells from SCNT-generated embryos appear equivalent to ES cells from fertilized embryos, how similar are iPS and ES cells? Undifferentiated iPSCs are molecularly indistinguishable from ES cells. However, differences between iPSCs and ES cells include low-degree chimeras resulting from iPSCs, increased tumor incidence with iPSCs, and the present inability to generate a full-term, all-iPSC mouse. Scientists have not yet succeeded at creating embryos using iPSCs to accomplish tetraploid complementation, in which all adult cells in the animal are derived from ES cells.

While iPSCs have great potential as sources of adult mature cells, much remains to be learned about the processes by which these cells differentiate. For example, iPS cells can give rise to cardiac cells that display action potentials; however, the maturation process into cardiomyocytes is impaired. Furthermore, great variation exists in the expression of markers in the iPSC-derived cardiac cells as compared to that seen in ES cell-derived cardiomyocytes. Therefore, iPSC-derived cardiomyocytes demonstrate normal commitment but impaired maturation, and it is unclear whether observed defects are due to technical or biological barriers. Thus, before these cells can be used for therapy, it will be critical to distinguish between iPSC-specific and disease-specific phenotypes.

Moreover, there appear to be subtle but detectable differences in methylation between ES and iPS cells (Deng J, et.al. *Nat Biotech* 2009;27:353-360). Reprogramming using an integrated retrovirus may confound the process, possibly through modulating endogenous genes or reactivating viral transgenes. Adenoviruses are currently being explored to generate iPSCs; these vectors offer the advantage of transient infection while still delivering the necessary transcription factors to promote reprogramming. While adenoviruses offer the possibility of genetically-pristine iPS cells, the efficiency of adeno-iPSC formation is several orders of magnitude lower than that of retroviral methods. Other strategies currently being explored to produce virus-free iPS cells include using small compounds (e.g., valproic acid, 5-aza-cytidine), protein transduction from recombinant proteins, non-integrating vectors (e.g., adenoviruses, non-integrating lentiviruses, episomal vectors), and excisable vectors such as transposons and floxed viruses. Each of these methods has advantages and disadvantages (Stadtfield M, Hochedlinger K. *Nat Methods* 2009;6:329-330).

Important questions in the iPSC/reprogramming field include:

- Why is reprogramming so inefficient and how can it be improved?
- Are ES cells and iPSCs truly equivalent?
- Do iPSCs derived from different donor cells behave differently? (e.g., do muscle-derived iPSC produce more muscle than skin-derived cells?)
- Can we establish novel disease models for degenerative disease that can be used for drug screening?

Dr. Hochedlinger concluded by observing that, in addition to answering these questions, researchers must study direct reprogramming into other cell types (e.g., screen for factors to “transdifferentiate” any cell type) and continue to explore alternative reprogramming strategies such as nuclear transfer.

#### Discussion:

Participants discussed the focus of future NIH funding to support iPSC research. It was noted that the development of disease models should be pursued in parallel with support for basic iPSC research. Although the reprogramming technology that creates iPSCs is currently imperfect, these cells will likely impact future therapy. Imperfect cells can illuminate many issues with therapy and regenerative medicine. While a disease-specific phenotype may be subtle and imposed by retroviruses, in some diseases, the phenotype is sufficiently strong that the retroviral influence will not matter. One attendee recommended considering whether the cells would be

used for cellular therapy or disease modeling because extensive characterization would be required for the former. Another attendee observed that iPS cells are not ready for applied cell therapy because of the potential for tumor formation. It was agreed that a significant gap exists between the lay understanding of the promise of iPS cells and the current level of understanding of the cells' biology, variability, and utility. Thus, an educational process is needed to make the public aware of the current state of the science and what will be required before the cells can be explored as therapeutic tools.

One attendee observed that the variability among ES cells may be even greater than that observed among iPSCs. Therefore, understanding the inherent variability among cell populations will be as important as understanding the differences between ES and iPS cells. As such, standardization of assays is critical; controls must be developed that account for variability in the iPS cells and their derivatives. It was also noted that models are being developed that will predict which cells will become iPSCs; the epigenetic state influences reprogrammability. The parallel differentiation of many iPSC and ES cell lines will also allow exploration of whether iPS cells carry a genetic "memory" of what they once were.

Another participant commented that the status of X-chromosome inactivation in human ES cells is not as clearly established as it is in mouse ES cells. The use of fibroblasts as sources of iPSCs suggests that modeling of X-chromosome diseases may be an avenue to pursue. Moreover, research should be carried out to explore the inactivation of the X chromosome in disease.

### **Session 2: Cardiovascular Disorders**

**Moderator:** John Thomas, Ph.D.; National Heart, Lung, and Blood Institute, NIH

#### ***Technologies for Association Studies and Diagnostics Using iPS Cells***

George M. Church, Ph.D.; Harvard Medical School

Dr. Church began by noting that there has been a ten-fold increase in genetic sequencing capacity per year since 2000, making sequencing a practical, cost-effective tool (e.g., a human diploid genome at 40x coverage (120 Billion base pairs of raw data) can be sequenced for \$5,000. Moreover, therapies can work in many instances even when the lesion of interest is present in an unassociated gene. The challenge of a systems biology approach, however, extends beyond translating genomic sequencing into therapy—*inherited and environmental genomics* must be considered on a regular basis. The Personal Genome Project ([www.personalgenomes.org](http://www.personalgenomes.org)) is an open-access database that combines a once-in-a-lifetime inherited genome sequences with yearly tests of allergens, microbes, viruses, and immune responses to these to establish a "bio-weather map" that can inform public health issues. Participants must provide an informed consent that does not promise de-identification, and users provide hair and skin cells that are reprogrammed to iPS cells and subsequently programmed to selected fates. Genome-wide bisulfite and enzyme assays are used to assess gene-body methylation (Ball MP, et.al. *Nat Biotech* 2009;27:361-368), and barcoding of miRNAs is employed extensively to enable high throughput (Vigneault F, et.al. *Nat Methods* 2008;5:777-779). Selective genome sequencing also allows capturing of alleles from genomic or c-DNA using *in vitro* paired tags (Shendure J, et.al. *Science* 2005;309:1728-1732), hybridization selection (Nilsson M, et.al. *Trends Biotechnol* 2006;24:83-88), or gap-filling (Porreca GJ, et.al.

*Nat Methods* 2007;4:931-936). To create oligomers en masse, chips can be used to produce as many as 15 million base pairs for as little as \$500 (Tian J, et.al. *Nature* 2004;432:1050-1054; Carr PA, et.al. *Nucleic Acids Res* 2004;32:e162; Smith J, Modrich P. *PNAS* 1997;94:6847-6850). Targeted sequencing technology continues to improve, enabling multiple hypothesis testing through pooling of select alleles by pathway and mutation type rather than linkage disequilibrium (LD) or chromosome position. As allelic and environmental changes occur, the number of hypotheses increases. Recent advances in technology also allow determination of the minimum number of hypotheses per individual per trait (e.g., three million if there is no linkage disequilibrium; 300/gene for cis-acting elements). Zinc finger nucleases enable highly-specific genome editing, facilitating the ability to establish causality via efficient allele replacement. By combining all cis-element variants, environmental and trans-acting variation among individuals can be eliminated (Forton JT, et.al. *Genome Res* 2007;17:82-87).

Clustering analysis of statistically significant allele-specific expression in reprogrammed cells reveals that approximately 50% of allele-specific expressions are invariant among cell types. Allelic expression switching is also observed in some instances. Dr. Church concluded by noting that genomic technologies applicable to iPS cells include barcoding, miRNA, RNA editing, methylation, exome and whole genome sequencing, and allele-specific expression.

#### Discussion:

Dr. Church noted that 5-10% of genes undergo allele switching; some of these have a cis-acting variation. He also observed that genome or transcriptome amplification becomes increasingly challenging as the amount of material to be amplified decreases, but has already surpassed by 1000-fold the 6 picograms required for single cell analysis. Some allelic expression is subject to stochastic variation, and it is not known how much of the low-level of transcription present at any given base pair is significant.

One participant commented that, while the accuracy of targeted sequencing is high, bias due to amplification is an issue that can be solved only through additional depth of coverage or minor amplification technology improvements.

Another attendee asked about the population scale necessary to establish a causative relationship between alleles and gene expression. Dr. Church replied that high-throughput allele replacement approaches (such as zinc-finger nucleases) establish causative relationships (of alleles and expression) even in very tiny cohorts (as low as N=1). Assessing expression to disease relationships (that will be meaningful to the individual) will benefit from cohorts of 10,000 or more.

#### ***iPS Cells for the Understanding and Treatment of Heart Disease***

*Deepak Srivastava, M.D.; Gladstone Institute of Cardiovascular Disease, University of California, San Francisco*

Dr. Srivastava began by noting that iPSCs offer opportunities to regenerate damaged tissue, to study the mechanisms of disease, and to develop patient-specific stem cells for screening and therapy. However, the derivatives of iPS cells must be well characterized before these cells can be used in clinical trials. He noted also that acquired heart disease is the leading cause of death,

causing 850,000 heart attacks per year. Currently, an estimated five million people in the United States have heart failure. Cardiac stem cell differentiation proceeds through several series of progenitor cells; disruption in any step results in heart disease. However, congenital heart disease is sufficiently common to map out detailed pathways that govern these diseases as they are developing (Srivastava D. *Cell* 2006;126:1037-1048). Creation of disease-specific iPS cell lines will inform researchers about the steps required of human progenitor cells as they adopt their fates. However, good reporters will be necessary at each step to track the changes.

Dr. Srivastava then noted that iPSCs may be able to adopt the necessary lineages for cardiogenesis. Fibroblasts from an adult cardiac-green fluorescent protein (GFP) mouse can be injected into an embryo to generate colonies of pluripotent stem cells. These iPS cell-derived cardiac cells are GFP-positive, and most can beat within a monolayer. At the level of gene expression, these cells are nearly identical to regular cardiac cells. It has been shown that miRNAs can guide differentiation and control differentiation away from unwanted lineages for all cell types that are used in the heart (Zhao Y, et.al. *Nature* 2005;436:214-220; Zhao Y, et.al. *Cell* 2007;129:303-317; Ivey KN, et.al. *Cell Stem Cell* 2008;2:219-229; Morton SU, et.al. *PNAS* 2008;105:17830-17835; Fish JE, et.al. *Dev Cell* 2008;15:272-284).

With regard to cardiac disease-specific applications of these cells, it has been shown that heterozygous mutations cause some autosomal dominant congenital heart disease (Garg V, et.al. *Nature* 2003;424:443-447; Garg V, et.al. *Nature* 2005;437:270-274). Several opportunities exist with regard to using disease-specific iPS cells, including using chromatin immunoprecipitation sequencing to interrogate epigenetic and promoter occupancy consequences in cardiac progenitors in humans with heterozygous transcription factor (TF) mutations. However, reporters will be needed to trace cell fates. In addition, RNA sequencing could correlate transcriptome consequences of human TF mutations in cardiac progenitors. Finally, disease-specific iPS cells could enable the tracing of cardiac progenitor fates within the setting of human TF mutations and heart malformations. Dr. Srivastava commended the National Heart, Lung, and Blood Institute (NHLBI) for several programs (e.g., the NHLBI Pediatric Cardiac Genetics Consortium, the NHLBI Pediatric Cardiac Development Consortium, and the NHLBI Progenitor Cell Consortium) designed to establish national networks of centers that will integrate iPS cells into their respective efforts.

As an example, Dr. Srivastava noted that aortic valve disease, the third most common form of heart disease in adults, is a congenital condition that evolves over decades. Aortic valve disease often results from a bicuspid aortic valve that may promote calcific aortic stenosis. It is known that mutations in NOTCH1, which directs cells away from an osteoblast fate, cause bicuspid aortic valves and calcific aortic stenosis (Garg V, et.al. *Nature* 2005;437:270-274). Cells from patients with NOTCH1 mutations and calcific aortic stenosis show a 50-fold increase in osteoblast-specific genes when cultured in calcification-promoting media, supporting the hypothesis that the condition could arise from a cell-fate switch from mesenchyme to osteoblast. Thus, human NOTCH1 iPS cells could theoretically be differentiated into cardiac cells that could inform the disease mechanism and be used as tools to screen drugs for aortic valve disease.

Dr. Srivastava concluded by noting that more than twenty cardiovascular disease-specific iPS cell lines are currently being generated. Much work is needed, however, to determine the variability of patient-specific iPS cell lines and to compare these cells with human ES cell lines.

Many commercial opportunities exist for these cells, including automation of technology, assay development for drug discovery, drug toxicity studies, and the development of cell-based therapies.

#### Discussion:

One attendee asked about the likelihood of generating a good cardiac phenotype and the number of lines that should be generated from each patient. Dr. Srivastava noted that the difficulty in modeling diseases will vary; therefore, researchers should consider which diseases could be reduced to a cellular level. He noted that his laboratory has generated several lines from each person in the immediate family of a patient with calcific aortic stenosis who has a NOTCH1 mutation.

He noted also that the null allele was affected in the family of the NOTCH1 patient and that transcription factor mutation is sufficient to cause disease. However, he stated that there is some evidence of cellular turnover in the heart, thereby suggesting a resident pool of stem cells. However, no consistent and accepted marker for these cells has been identified.

#### ***Challenges for iPS Cell-Based Therapies for Treating Cardiovascular Disease***

*Joseph C. Wu, M.D., Ph.D.; Stanford University*

Dr. Wu began by reviewing several considerations for using iPS cells to treat cardiovascular disease. Since patients with ischemic heart disease have high a mortality rate, rapid and safe reprogramming strategies must be developed. Since iPSCs also have teratogenic potential, novel technologies must be developed to track cell fate *in vivo* for pre-clinical and clinical trials. While iPS cells may allow researchers to create a bank of phenotypes, it is essential to determine whether these cells will be rejected and if immunosuppressive drugs can be used to further augment tolerance. Since iPS-differentiated cardiac cells will most likely come from people who have preexisting conditions, it will be critical to determine if the cells' efficacy will be similar to that of ES cells. Several novel strategies are currently being explored to reprogram iPS-derived cardiac cells, including using a fat-cell source to improve yield, utilizing non-immunogenic, non-viral "minicircle" DNA vectors, and characterizing the functionality of iPS cell-derived cardiomyocytes (iPSC-CM) in small and large animal models of human disease using state-of-the-art molecular imaging technology.

Human adipose stromal cells (hASCs), an abundant source of fat tissue, can be harvested during liposuction. These cells are much easier to reprogram than are fibroblasts, suggesting that these cells may be one source for generating iPS cells quickly and efficiently. Colonies of iPS cells are generated from hASCs within 16 days, with ~20-fold increase in the number of colonies relative to fibroblasts (Sun N, et.al. *PNAS* 2009; in press). These iPS colonies can be derived on Matrigel plates, and the cells' methylation pattern resembles that of undifferentiated human ES cells. Preliminary data suggest that these cells are also amenable to reprogramming using non-viral "minicircle" vectors. These vectors are created using phage C31 integrase to mediate intra-molecular recombination between attB and attP sites, thereby creating a minicircle and bacterial backbone (Huang M, et.al. *Circulation* 2009; in press). Another approach is to use microRNAs (miRNAs), which are an endogenous class of small, non-coding RNAs that are important for human ES cell self-renewal, pluripotency, and differentiation (Wilson KD, et al. *Stem Cells &*

Development 2009;18(5):749-757), provide “signatures” of iPS cells that will provide insight into the reprogramming pathways. Dr. Wu noted that as the field moves forward, issues of epigenetic stability and reproducible differentiation will be critical to generate and transplant iPS cell-derived cardiomyocytes (Swijnenburg RJ, et.al. *Curr Opin Biotechnol* 2007;18:38-45).

Once injected, iPS cells may form teratomas, and recipients may require immunosuppressive therapy. However, many molecular imaging modalities, including microPET, audioradiography, ultrasound, bioluminescence, and microCT, can be used to track cells *in vivo*. Imaging of ES cells *in vivo* can reveal patterns of survival and immune rejection following transplantation (Swijnenburg R-J, et.al. *Stem Cells Dev* 2008;17:1023-1030; Swijnenburg R-J, et.al. *PNAS* 2008;105:12991-12996). Dr. Wu noted that although Geron Corporation has received clearance to begin a trial of human ES cell-derived oligodendrocyte progenitor cells for the treatment of acute spinal cord injuries, it is presently unclear whether the cellular products or the immunosuppressive protocol used to prevent rejection will be safe and effective.

Detection of teratoma formation will also be a critical area of research to support future iPS cell-based therapy trials. Tumors in humans express high levels of  $\alpha_v\beta_3$  integrins (Cao F, et.al. *Cancer Res* 2009;69:2709-2713), suggesting possible gene therapy approaches using the suicide- and PET reporter gene, HSV-tk (Cao F, et.al. *Circulation* 2006;113:1005-1014). HSV-tk serves as a suicide gene when ganciclovir is administered in milligram doses. In the mouse model, teratoma formation can be seen with as few as 10,000 to 100,000 undifferentiated human ESCs (Lee A, et al. *Cell Cycle* 2009;8(16):1-5). On the other hand, transcriptional and functional profiling of human ES cell-derived cardiomyocytes indicates poor long-term cell survival following injection; most cells die within three months (Cao F, et.al. *PLoS ONE* 2008;3:e3474). Thus, the problems of teratoma formation with undifferentiated cells and acute donor cell death with fully differentiated cells will continue to hamper efforts for regenerative medicine based on human iPS cells and ESCs.

Dr. Wu concluded by observing that additional issues still remain to be resolved, including deriving iPS cell lines from patients with hypertrophic cardiomyopathy and idiopathic dilated cardiomyopathy, establishing genetic and functional phenotypes of iPSC-CMs, and deriving iPS cell lines from large animal models. He observed that hESC-CMs resemble neonatal cardiomyocytes rather than adult cardiomyocytes that have been subjected to mechanical stresses. He also noted that there may be issues with respect to industrial providers because of the difficulties associated with developing a standardized product that incorporates individual variability.

#### Discussion:

One attendee asked whether variability in circulation could be controlled for when imaging cells. Dr. Wu replied that little variability is observed, and techniques such as bioluminescence offer the additional advantage of signal-free probes. He noted also that teratoma formation is related to the environment; for example, cell growth appears more rapid following injection into the kidney and liver than in the leg and heart.

Another participant asked about miRNA expression with minicircle vectors. Dr. Wu noted that miRNAs are transiently expressed with non-viral minicircle vectors, although it has not been

quantified how these miRNA levels change. Following *in vivo* delivery, miRNA expression lasts for 6-8 weeks, although it drops rapidly within the first week. However, at present, it is not clear how long miRNA expression lasts in a cell following *in vitro* cell transfection with minicircles.

### **Session 3: Nervous System**

**Moderator:** David Owens, Ph.D.; National Institute of Neurological Disorders and Stroke, NIH

#### ***Using iPS Cells to Study the Cell Biology of Autism***

Ricardo Dolmetsch, Ph.D.; Stanford University

Dr. Dolmetsch began by stating that iPS cells may provide insight into the biology of neurodevelopmental diseases, noting that researchers currently do not understand the cellular phenotypes of mental disorders. Autism was first described in 1943 by Leo Kanner, who characterized the disorder in children who exhibited difficulties in social interactions, repetitive movements and activities, and difficulty producing and interpreting language. Autism is a spectrum of diseases that has a wide variety of underlying pathological causes. These disorders have a strong genetic basis--concordance between identical twins is 90% and between siblings is 10% (Folstein S, Rutter M. *Child Psychot Psychiat* 1977;18:297-321). Three genetic models of autism have been proposed, in which the diseases are caused by many rare alleles, a combination of common alleles, or a combination of rare and common alleles. Most likely, autism is caused by a relatively penetrant *de novo* mutation conferred on a background that promotes susceptibility. Many genes have been associated with autism, all of which are associated with other syndromes.

For many reasons, iPS cells may prove a convenient and useful tool to study autism. Genes alter cell biology and function, which affect circuits and ultimately, behavior. Autism is multigenic, so it is not suited to a mouse model. Many mutations associated with autism are not penetrant, yet the genetic background appears important (parents of autistic children often present with some autistic symptoms). Furthermore, human and mouse brains diverged 60 million years ago and differ at the cellular level (Povysheva NV, et.al. *J Neurophysiol* 2008;100:2348-2360).

One approach to using iPSCs to study autism involves harvesting skin cells from patients with neurodevelopmental disorders and reprogramming them to iPS cells using the methods developed by Yamanaka and colleagues (Takahashi K, et.al. *Cell* 2007;131:861-872). The pluripotent stem cells can then be converted into a broad set of neurons phenotypic of the disorders. Although it is unclear how to phenotype these neurons, defects in calcium signaling have been identified using a variety of high-content assays, including calcium and excitability, migration of neurons, morphology (dendrite formation), and synapse formation. Patients can be selected based on their having Timothy syndrome or other autism-associated genes (e.g., Ch22q13, Shank3).

Dr. Dolmetsch noted that searching for cell-intrinsic neuronal defects in patients with autism poses challenges. Increased brain size, defects in columnar organization and dendritic structure, and epilepsy each have defects that may be cell-intrinsic. A mouse model of Timothy syndrome, which features relatively cell-intrinsic defects such as retraction of dendrites and defects in cell migration, has been developed to study cell-intrinsic defects in neurons. iPS-derived neurons are

also being compared with neurons harvested surgically to determine similarity. The iPS-derived neurons appear immature (e.g., from a child rather than an adult).

Questions that must be answered before iPSCs can be used to phenotype patients on a significant scale include:

- How many iPS cell lines are required per patient? (three to four are being generated in the experiments described here)
- How extensively should each line be characterized (at an approximate cost of \$10,000/line)?
- Does it matter whether the lines contain integrated viruses?
- Is a pure population of neurons necessary or are single-cell assays sufficient?
- Which phenotypes of iPS-derived neurons are robust?

#### Discussion:

To avoid duplication of effort and to promote standardization, one attendee suggested that the NIH consider supporting a few Centers that would generate iPS cells and reagents that could then be distributed to laboratories. Such a centralized facility could also generate iPS-derived neuronal precursors and other cells that would support validation of derivatives generated by users.

#### ***Human iPS Cells and hESCs for the Central Nervous System (CNS)***

*Fred H. Gage, Ph.D.; Salk Institute for Biological Studies*

Dr. Gage began by observing that human ES cells can differentiate into motor. However, 1-2 months in culture are necessary for ES cells to differentiate into authentic neurons that express motor-neuron genes, innervate muscle, and become physiologically active (e.g., form neuromuscular junctions and synapses and fire action potentials). Activation of astrocytes is a common feature of neurological disorders and a potential mechanism for cell-non-autonomous death. Derived motor neurons co-cultured with iPS-derived cells may provide an approach to screen phenotypes for drug development.

Another strategy involves transplanting human ES cells into mouse embryos *in utero* and characterizing the cells once the pups have matured. Reprogramming of human fibroblasts into functional neurons using the retroviral technology developed by Yamanaka and colleagues results in heterogeneous colonies after 15 days. The iPS cells form neural rosettes in culture and differentiate into neurons. As individual cells, these iPSCs are electrophysiologically active. A lentiviral-based approach may also be used to generate neurons, thereby affording the advantage of a transient expression period. Other cell types such as keratinocytes and neuroprogenitors can be used to make iPS cells and, ultimately, neurons. Viral-free neuroprogenitor-iPS cells can be reprogrammed. iPS cells are similar across lines, as are human ES cells, but somewhat different from each other. Clustering analysis suggests that some genes are involved in the reprogramming process. In conclusion, Dr. Gage observed that iPSCs are similar to human ES cells and differentiate into neurons readily. Although some genetic differences have been identified, there is more similarity across lines of iPS cells than would be expected.

Discussion:

One attendee asked if calcium currents had been explored in different subsets of cells as a way to investigate the cellular basis of psychiatric disorders. It was noted that it will be critical to learn how to differentiate cells into a potential phenotype that mimics central nervous system behavior *in vivo*. The effects of stresses will be more readily apparent as the complexity of the physiologic environment is replicated. One attendee commented that a defined population of cells is a critical tool for these studies; an understanding of the disease will help to select a relevant patient population for screening. iPS-derived non-neural cells will also be important to understand the nature of CNS disease and to support disease screening.

**Session 4: Diabetes**

**Moderator:** Sheryl Sato, Ph.D.; National Institute of Diabetes and Digestive and Kidney Diseases, NIH

**Human Stem Cells to Understand and Develop New Treatments for Type 1 Diabetes**  
Douglas Melton, Ph.D.; Harvard University

Dr. Melton began by observing that type 1 diabetes is a complex, multigenic disease, the root cause of which remains unknown. Treatments, which are largely based on studies of non-obese diabetic (NOD) mice, are non-specific and carry side effects. Donor tissue for transplantation is limited, and a cure of diabetes is absent. Moreover, many therapies that have proved successful in NOD mice fail in humans (von Herrath M and Nepom GT. *Nat Immunol* 2009;10:129-132). In type 1 diabetes, beta cells are eventually lost through an unknown pathway or pathways. It is not known which cell type must possess the diabetic genotype for a person to acquire the disease. Type 1 diabetes reflects a failure of proper self/non-self education in the thymus; however, the initial cause of this failure is not understood. Human iPS cells can be used to investigate the root cause of the disease, as these cells could be reprogrammed into the thymic epithelial cells, blood cells, and beta cells that are implicated in disease progression. Given the importance of generating iPS cells efficiently and reliably, Dr. Melton suggested that the NIH consider funding core laboratories to produce iPS cells.

Dr. Melton also noted that more than 75 human ES cell lines and more than ten diabetic iPS cell lines have been derived at the Harvard Stem Cell Institute. Directed differentiation of iPS or ES cells to beta cells proceeds through a developmental pathway that includes definitive endoderm, foregut, pancreas, and endocrine cells. To recreate the first step in this sequence, two compounds can be used to establish definitive endoderm. Current data suggest that compound-induced endoderm has a similar gene expression profile to its native counterpart *in vivo*. These compounds can differentiate approximately 80% of iPS or ES cells into endoderm (Borowiak M, et.al. *Cell Stem Cell* 2009;4:348-358). A second compound, indolactam V, can transform 45% of foregut cells into pancreatic cells (Chen S, et.al. *Nature Chem Biol* 2009;5:258-265). Although small molecules will likely be one tool to create a beta cell, genetic reprogramming using hepatocytes or acinar cells represents another viable approach. For example, diabetic patient-specific iPS (DiPS) cells have been created using the Yamanaka method that behave like human ES cells and are pluripotent by all assays. To recapitulate human type 1 diabetes in a humanized

mouse, DiPS cells must be differentiated into thymic epithelial cells, blood cells, and pancreatic beta cells.

Thymic epithelial cells are necessary to understand T-cell selection. The thymus derives from a single endodermal origin (Gordon J, et.al. *Nat Immunol* 2004;5:546-553). A common progenitor exists for thymic cortical and medullary epithelium (Rossi SW, et.al. *Nature* 2006;441:988-991), and a postnatal epithelial progenitor cell can initiate the formation of a functional thymus (Bleul CC, et.al. *Nature* 2006;441:992-996).

Dr. Melton concluded by noting several challenges for diabetes stem cell projects, including:

- Creating pluripotent ES and iPS cell lines (e.g., cell sources, methods and culture conditions, and large-scale production to account for variation in population)
- Directing differentiation of stem cells—chemical screening may be more important than co-culture with growth factors, given differences between mice and humans
- Creating a humanized mouse to develop functional tests for development, physiology, and pathology.

He noted also that the Beta Cell Biology Consortium ([www.betacell.org](http://www.betacell.org)) has been especially useful as a support resource for this work. The Consortium supports larger, group-oriented projects that require shared reagents.

***Studying Type 1 Diabetes by Combining Human iPS and Humanized Mouse Technologies***  
*Dale Greiner, Ph.D.; University of Massachusetts Medical School*

Dr. Greiner began by describing humanized mice, which are mice that are engrafted with functional human cells or tissues or that express human transgenes. These mice, which can be used as tools to help realize the potential of iPSCs, serve as vessels to test the functionality and tumorigenicity of cell populations. Mouse models used for human regenerative medicine have shown that tissue injury or loss is important in providing a niche with appropriate signals for stem-cell regeneration. Immunodeficient mice that receive iPS cells can be used to study type 1 diabetes by combining human iPS cells from diabetic donors with humanized mouse technologies. Short-term goals for this approach include the development of a functional immune system, hyperglycemia to evaluate beta cells derived from stem and progenitor cells, and an appropriate engraftment model system. Existing mouse models proved insufficient for the study of human beta stem/progenitor cells because NOD-*scid* mice have short lifespans and natural-killer cell activity (Tian X, et.al. *Stem Cells* 2006;24:1370-1380; Sotiropoulou PA, et.al. *Stem Cells* 2006;24:74-85; Greiner DL, et.al. *Stem Cells* 1998;16:166-177; Shultz LD, et.al. *J Immunol* 1995;154:180-191). Moreover, these mice cannot be engrafted with a functional immune system.

However, gamma-chain knockout mice are on a NOD background and thus can engraft with human tissues. Immunodeficient NOD mice bearing a mutation in the interleukin (IL)-2 receptor common gamma chain lack adaptive immunity and have severe defects in innate immunity (e.g., long life span, no NK cells). These developments have enabled the current “state-of-the-art” model, the NOD-*scid* *IL2 $\gamma$ <sup>null</sup>* (NSG) mouse (Shultz LD, et.al. *Nat Rev Immunol* 2007;7:118-130). NSG mice can be engrafted with a functional immune system. Limitations of human

engraftment in NSG mice include a lack of human leukocyte antigen (HLA) molecules required for appropriate T-cell function, species specificity of growth factors and other molecules, and some remaining innate immunity.

Four HLA supertypes--HLA clusters based on a shared sequence motif in peptide-binding pockets—are currently being put on a NOD-*scid* background. It is estimated that these four HLA-transgenic strains will cover approximately 77% of persons with type 1 diabetes, including many African-Americans, Caucasians, Asians, and Hispanics. NSG mice are currently being developed that reflect selection of several growth factors and other molecules (e.g., stem cell factor, BlyS, IL-2, IL-7, IL-15,  $\text{Sirp}\alpha$ ,  $\text{LT}\alpha$ ). However, it has been reported that human ES cell-derived progenitor cells form pulmonary emboli following intravenous injection into NOD-*scid* mice (Wang L, et.al. *J Exp Med* 2005;201:1603-1614).

Dr. Greiner then noted several basic questions in beta-cell biology, including:

- What is the number of insulin-positive cells are required to restore normal glycemia in diabetic NOD-*scid*  $IL2\gamma^{null}$  mice?
- Can NOD-*scid*  $IL2\gamma^{null}$  mice be used to determine the function of human ES cell- or iPS cell-derived beta stem and progenitor cells?
- Can human beta cell proliferation/regeneration be studied in NOD-*scid*  $IL2\gamma^{null}$  mice?

Preliminary experiments indicate that transplantation of 500,000 dissociated mouse insulin-positive beta cells restores normoglycemia. However, transplanted human cells do not appear to be as successful at restoring normal glycemic levels. Human islets transplanted into pregnant NOD-*scid*  $IL2\gamma^{null}$  mice were analyzed on day 21 of gestation following pulsing with BrdU in the drinking water for 6-7 days. Controls are currently being analyzed, but little proliferation has been observed.

Four models of hyperglycemic NSG mice are available that are recipients of human beta stem/progenitor cells and have a functional immune system, including models that have been treated with streptozotocin, diphtheria toxin, and tetracycline-doxycycline. In addition, an “Akita” model of spontaneous hyperglycemia without autoimmunity is available in which a non-autoimmune diabetes will develop as the mouse ages in the absence chemical treatment that could harm the transplanted beta stem/progenitor cell or human immune system. The Akita mutation is an insulin-2 gene defect that leads to generation of mis-folded insulin protein, induction of endoplasmic reticular stress, beta-cell apoptosis, and a non-autoimmune hyperglycemia. Human hematopoietic stem cells engraft in hyperglycemic Akita mice and generate a functional immune system, and human islets restore normoglycemia in these mice.

To develop an appropriate engraftment system for beta cells, hematopoietic stem cells, and human fetal thymus, the SCID-Hu model is used (McCune JM, et.al. *Science* 1988;241:1632-1639). This model allows the introduction of an intact human immune system and pancreas to ascertain whether the immune system destroys the organ. Current experiments are investigating the simultaneous addition of fetal tissues as a proof-of-principle. The long-term goal of this work is to develop mouse models in which each component affected by type 1 diabetes is replaced with iPS-derived tissues.

Discussion:

It was noted that some human red blood cells are formed when the immune system is reconstituted. Dr. Greiner noted that this mouse model has been used to deliver small interfering RNA to diseases such as ebola and HIV. The model can be expanded to other autoimmune diseases, and the general approach is applicable to type 2 diabetes.

One attendee asked about the percentage of human lymphocytes seen in these mice, and Dr. Greiner noted that as much as 90% of circulating cells appear to be human. Although the response is not as robust as it could be, these models feature fairly robust human immune systems with T-dependent and T-independent immune responses.

***Panel Discussion***

Suggestions that emerged from this workshop include:

- Establish Centers to create and distribute disease-relevant iPS cells. It was suggested that these Centers would distribute the cells freely before awaiting publications. It was estimated that five such locations are sufficiently qualified at present to carry out such work. It is recognized that the core technology will evolve with appropriate methodologies; however, Centers could agree *a priori* to use a common platform. One attendee stated that failure to establish these Centers could result in duplicative efforts by R01-funded investigators who would generate cells that may be poorly characterized. These Centers would not serve as central banks that collect cells from investigators.
- Consider forming consortia modeled on the Beta Cell Biology Consortium
- Explore the utility of the mouse vessel for some diseases
- Grandfather all published cell lines
- Consider biobanking iPS cell starting materials, such as skin fibroblasts. This approach would require a clear informed consent policy. It was also suggested that the NIH could consider requiring that associated data be deposited along with the materials, although one participant noted that such a policy would be difficult to implement.
- Consider small business programs as a way to engage potential industrial partners

Although iPS cells hold great promise for advancing basic science understanding of development and for treating numerous diseases, the field is in its infancy. Although many advances are necessary before the technologies can find clinical application, iPS cells are immediately useful as vehicles for discovery of new molecular pathways. These cells will also be useful to identify small molecules for drug development. As such, a focus on the use of iPS cells in transplantation therapy is premature at present. Therefore, over the next two or three years, the emphasis on transplantation therapy should be modulated with the cells' value for illuminating the pathophysiology of disease at the molecular level and for replacing cells that have been damaged by disease.

Several attendees likened this field's current dynamic to that of the early days of the Human Genome Project (HGP); however, it must be noted that much remains to be done to achieve the level of standardization seen with the HGP. In its earliest stages, HGP researches had yet to

establish the Centers and core facilities that ultimately standardized and codified research efforts. It was agreed that establishment of these Cores did not stifle innovation or independent experimentation.

***Closing Remarks and Summary***

*Story C. Landis, Ph.D.; National Institute of Neurological Disorders and Stroke, NIH*

Dr. Landis thanked the speakers and organizing committee for an excellent and thought-provoking set of discussions.

The meeting was then adjourned.